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Sex allocation plasticity on a transcriptome scale: socially-sensitive gene expression in a simultaneous hermaphrodite

Running title: Sex allocation plasticity in hermaphrodites

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Abstract

Phenotypic plasticity can enable organisms to produce optimal phenotypes in multiple environments. A crucial life history trait that is often highly plastic is sex allocation, which in simultaneous hermaphrodites describes the relative investment into the male versus female sex functions. Theory predicts – and morphological evidence supports – that greater investment into the male function is favoured with increasing group size, due to the increasing importance of sperm competition for male reproductive success. Here we performed a genome-wide gene expression assay to test for such sex allocation plasticity in a model simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. Based on RNA-Seq data from 16 biological replicates spanning four different group size treatments, we demonstrate that at least 10% of the >75,000 investigated transcripts in *M. lignano* are differentially expressed according to the social environment, rising to >30% of putative gonad-specific transcripts (spermatogenesis and oogenesis candidates) and tail-specific transcripts (seminal fluid candidates). This transcriptional response closely corresponds to the expected shift away from female and towards male reproductive investment with increasing sperm competition level. Using whole-mount *in situ* hybridization, we then confirm that many plastic transcripts exhibit the expected organ-specific expression, and RNA interference of selected testis- and ovary-specific candidates establishes that these indeed function in gametogenesis pathways. We conclude that a large proportion of sex-specific transcripts in *M. lignano* are differentially expressed according to the prevailing ecological conditions, and that these are functionally relevant to key reproductive phenotypes. Our study thus begins to bridge organismal and molecular perspectives on sex allocation plasticity.

Keywords

Gene expression; Oogenesis; Phenotypic plasticity; Sex allocation; Spermatogenesis; Sperm competition

Introduction

Predicting an organism's optimal sex allocation has long been a central concern of evolutionary biology (Darwin 1871; Düsing 1884; Fisher 1930; Hamilton 1967; Charnov 1982; West 2009), and the widespread empirical support for sex allocation theory is often heralded as one of the great success stories in predicting adaptive evolution (e.g. West *et al.* 2000; Frank 2002). Although there is a strong bias in the sex allocation literature towards studying sex ratios in separate-sexed taxa (Hardy 2002; West 2009), there is now accumulating experimental support for sex allocation adjustments also in sequential and simultaneous hermaphrodites (Munday *et al.* 2006; Schärer 2009).

In simultaneous hermaphrodites, the question of optimising sex allocation means predicting the balance of resources invested into an individual's male versus its female sex function (Charnov 1979; 1982; 1996). Theory indicates that individuals should shift investment more towards the male sex function as the mating group size (i.e. the number of mating partners plus one) increases (Charnov 1982), due to the increasing importance of success in sperm competition in determining fitness gains through the male sex function. This is thought to favour the evolution of phenotypically plastic sex allocation strategies that can respond to relevant environmental cues of mating group size (Schärer 2009) – such as social group size (Janicke *et al.* 2013) – for which there is strong empirical support (Trouvé *et al.* 1999; Schärer & Ladurner 2003; Tan *et al.* 2004; Brauer *et al.* 2007; Schärer & Janicke 2009; Schleicherova *et al.* 2010; Al-Jahdali 2012; Janicke *et al.* 2013). Because in this manner they

can, within their own lifetime, both adjust their sex allocation and receive the fitness payoffs of doing so, this makes simultaneous hermaphrodites especially valuable and unique models to test sex allocation theory.

Despite this body of evidence substantiating sex allocation theory from a morphological perspective, the genetic details of how sex allocation plasticity is achieved remain poorly understood (Pannebakker *et al.* 2011). To begin to address this for the case of simultaneous hermaphrodites, we performed an RNA-Seq experiment in *Macrostomum lignano*, a free-living, simultaneously hermaphroditic flatworm (Ladurner *et al.* 2005b). This species represents an excellent model for studying the genetic basis of sex allocation plasticity, because it is known to respond to relevant social cues by plastically adjusting its allocation to sperm and egg production. At larger group sizes, worms exhibit larger testes (Schärer & Ladurner 2003; Brauer *et al.* 2007; Janicke *et al.* 2013) containing more active spermatogenic cells (Schärer *et al.* 2004b) and exhibiting a faster rate of spermatogenesis (Giannakara *et al.* 2016), and so have a higher sperm production rate (Schärer & Vizoso 2007). Responses in ovary size tend to be more variable, but it is typically either reduced (Schärer *et al.* 2005) or sometimes unaffected (Schärer & Ladurner 2003; Janicke *et al.* 2013) at larger group sizes. Such a relative shift towards the male sex function with increasing group size is in close agreement with predictions from sex allocation theory (Janicke *et al.* 2013). Moreover, this shift has been shown to represent a phenotypically flexible response, i.e. even adult worms moved to a new social environment can rapidly adjust their sex allocation to suit the prevailing conditions (Brauer *et al.* 2007).

By raising individuals in different social environments (group sizes: isolated, pairs or octets), predicted to lead to different optimal sex allocation patterns, we here aimed to uncover the transcriptional landscape of sex allocation plasticity. For selected candidate transcripts, we then followed this up with functional characterizations using a combination of *in situ* hybridization and RNA interference experiments.

Materials & Methods

Study organism

M. lignano is a transparent, outcrossing simultaneous hermaphrodite that reaches ca. 1.5mm in length as an adult. Worm cultures are maintained in glass Petri dishes filled with f/2, a nutrient-enriched artificial seawater (32‰ salinity), and fed with diatoms (*Nitzschia curvilineata*), on a 14:10 light:dark cycle at 20°C and 60% relative humidity (Rieger *et al.* 1988; Ladurner *et al.* 2005b). All animals in this experiment were taken from a culture of the highly inbred DV1 line (Janicke *et al.* 2013) that has also been used to generate the genome and transcriptome assemblies for this species (Wasik *et al.* 2015; Wudarski *et al.* 2017).

Group size experiment

We began by confirming sex allocation plasticity at the morphological level for our experimental subjects. To generate experimental subjects of standardized age, ca. 1100 adult parental DV1 worms were distributed among 12 Petri dishes containing 20ml f/2 and a dense covering of algae, and allowed to lay eggs. Three days later, all parental worms were transferred to a second batch of Petri dishes prepared in the same way and then three days later the parentals were removed. We harvested the resulting hatchlings from each batch of

dishes ten days after the adults were introduced. Development time from egg laying to hatching in *M. lignano* is around five days, meaning that the expected age of the hatchlings at the start of the experiment was 2-5 days.

On the first day of the experiment (day 0), 1016 hatchlings were allocated randomly to one of four different treatment groups, representing different social contexts expected to lead to differential sex allocation: *isolated* (n = 224 individuals) were kept alone for the duration of the experiment; *joined* (n = 224 individuals) were kept alone for the duration of the experiment, except that for the final 24h always two worms were paired together (aimed at studying short-term responses to mating); *pairs* (n = 264 individuals, in 132 groups), in which two worms were kept together for the duration of the experiment; and *octets* (n = 304 individuals, in 38 groups), in which worms were kept together in groups of eight for the duration of the experiment. All experimental groups were constituted in single wells of 24-well plates (TPP, Trasadingen, Switzerland) containing 1.5ml f/2 and fed with a dense algae suspension, the amount of which was adjusted to equalize per capita food availability across the different treatment groups, which was nevertheless expected to be *ad libitum*. In total, this required 28 x 24-well plates, with treatment groups balanced across plates and for position within plates. Worms were transferred to fresh wells every 7-11 days (five transfers in total). On day 63, *joined* worms were paired with a randomly chosen individual from the same treatment group on the same plate.

On day 64, a subset of 37-38 worms per treatment group was randomly selected (taking maximally one worm per well for the *pairs* and *octets* to ensure statistical independence) for morphological assessment of sex allocation (see next section). To then sort the worms into independent biological replicates for downstream analysis, we split the 28 plates used during the group size experiment into four independent batches, and then within each batch we

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pooled all of the worms belonging to the same treatment group (i.e. all worms from the same treatment and batch were pooled into just one replicate). In total, we thereby produced 4 x 4 independent biological replicates for the gene expression measurements. Owing to slight differences in plate contents and a low level of background mortality normally observed in our worm cultures (here around 6%), each biological replicate differed slightly in the number of worms it contained, from 49-76 individuals (median: 57 individuals). Note that in the case where an individual died in the *isolated*, *joined* or *pairs* treatments, the whole replicate was lost. However, to avoid losing large numbers of groups, in the case of the *octets* the loss of a single worm per well was tolerated (in 6/38 groups), so that the remaining seven worms in these wells could still be used. We reasoned that a group size of seven would still predict a much more male-biased sex allocation than the next closest group size of two, as has been shown (Janicke *et al.* 2013). Whole worms for each replicate sample were combined and homogenized using a bead beater in 700µl of cold (4°C) TRIzol® Reagent (Invitrogen) and stored at -80° until RNA extraction.

Morphological assay of sex allocation plasticity

In order to estimate the relative investment into the male vs. the female sex function, the testes and ovaries of worms were measured using standard techniques for this species (Schärer & Ladurner 2003). Briefly, worms were relaxed using a solution of MgCl₂ and then squeezed on a microscope slide to a standard thickness using spacers, and photomicrographs then obtained for organs of interest (testes, ovaries) at 400x magnification using a Leica DM2500 microscope and a digital Firewire camera (model DFK 41BF02; The Imaging Source Europe GmbH, Bremen, Germany) in combination with the image capture software BTV Pro (Ben Software). Due to the standardized squeezing, we expect that the two-dimensional images thereby obtained correspond well to the tissue volume of both the testes and ovaries. Images were later processed with ImageJ (<http://rsb.info.nih.gov/ij/>) to obtain

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estimates of testis area and ovary area for each worm, from which we estimated sex allocation as testis area / (testis area + ovary area) (Suppl. File S1). These measures of testis and ovary area are highly repeatable and have been employed in numerous previous studies in *M. lignano* to compare sex allocation under different experimental conditions (e.g. Schärer & Ladurner 2003; Schärer *et al.* 2004b; Brauer *et al.* 2007; Schärer & Vizoso 2007; Janicke *et al.* 2013; Giannakara *et al.* 2016)).

Total RNA extraction and library construction

Having shown that our experimental worms responded to their prevailing social environment by altering their sex allocation in agreement with our expectations (i.e. that they shift investment towards the male sex function with increasing group size, see ‘Worms adjust their sex allocation to the prevailing social conditions’ in the Results section), we next investigated the transcriptional basis of this response. Based on the four replicate RNA pools per treatment, we generated a total of 16 independent cDNA libraries for further transcriptomic analysis (i.e., four per treatment group). Total RNA was extracted from whole worms using a standard chloroform extraction protocol, re-suspended in 100µl of nuclease free water, treated with RQ1 RNase-Free DNase (Promega) following the manufacturer’s protocol and finally re-suspended in 20µl of nuclease free water after removing the DNase (i.e. a standard TRIzol extraction protocol for liquid samples). Quantitation assays using the Qubit (picogreen) were in the range 590-1400 ng, except for one sample assessed at 330 ng; for all other samples, the amount of RNA starting material was equalized to 500 ng prior to library construction. Library construction and subsequent NGS was performed by Fasteris SA (Plan-les-Ouates, Switzerland) using their standard (Illumina) mRNA protocol. Note that all libraries are based on extractions from whole worms, such that the resulting read counts tell us about overall differences in expression between treatments (our primary research question), but not directly – in the case of tissue-specific transcripts – about whether such

changes stem from shifts in tissue volume and/or expression per unit volume (although we explore this question in the ‘Genomic reaction norms for gametogenesis’ section in the Results).

Next generation sequencing and mapping

Following cDNA library construction and multiplexing, NGS sequence data were obtained from one lane of a HiSeq 2000, sequencing 100bp single-end reads (Fasteris). Sequencing reads were mapped to the *M. lignano de novo* transcriptome assembly MLRNA110815 (<http://www.macgenome.org/download/MLRNA110815/>) for consistency with previously published work (Arbore *et al.* 2015). MLRNA110815 is an early version of the reference *de novo* transcriptome assembly MLRNA150904 (Grudniewska *et al.* 2016), and it was assembled and annotated in a similar way, but was based on a smaller RNA-seq dataset. To ensure the maximal diversity of transcriptome sampling, the libraries used for the transcriptome assemblies were made from mixed populations of animals containing juveniles at different stages of development and adult hermaphrodites of different ages. The assemblies are therefore expected to contain all male- and female-specific transcripts of interest in this study. Sequencing reads were mapped to the MLRNA110815 assembly using bowtie v. 0.12.7 (Langmead *et al.* 2009) with parameters “-n 2 -e 99999999 -l 16 -a -m 200 -best -strata”. Mapping yielded 132,607,805 mappable reads, with at least one read mapping to 74,211 of a total of 76,437 transcripts (= 97%) in the MLRNA110815 assembly; per sample, we obtained between ca. 7.3 to 10.1 x 10⁶ mappable reads, with coverage between ca. 79-85% of the total transcriptome (Suppl. Table S1). Next, transcript expression levels were derived from the read mapping data by RSEM v1.1.19 (Li & Dewey 2011), which accounts for read mapping ambiguity.

For assigning transcripts from the *de novo* transcriptome assembly MLRNA110815 to the most recent genome-guided transcriptome assembly Mlig_RNA_3_7_DV1_v3 (Wudarski *et al.* 2017), sequences were mapped by blast with the parameters “-W 30 -e 0.01” in both directions (i.e. MLRNA110815 vs. Mlig_RNA_3_7 and Mlig_RNA_3_7 vs. MLRNA110815) and the single best hits from both searches were merged. Functional annotations were then extracted from the genome-guided transcriptome assembly Mlig_RNA_3_7_DV1_v3 (Wudarski *et al.* 2017); these include homologs from human (GRCh37), *Drosophila melanogaster* (BDGP), *Caenorhabditis elegans* (Wormbase) and *S. mediterranea* (Brandl *et al.* 2016) identified using blastx v.2.2.6 (Altschul *et al.* 1997) and taking the best hits with e-value cutoff below 0.01, plus Pfam domains from the Pfam database v. 27 (Finn *et al.* 2016).

Differential expression analysis

We identified differentially expressed candidates using the programs *DESeq* (Anders & Huber 2010) and *baySeq* (Hardcastle & Kelly 2010). The two approaches are complementary, with the former permitting pairwise comparisons between the two most contrasting treatments groups, i.e. *isolated* vs. *octets*, thereby identifying the maximum number of transcripts as putative differentially expressed candidates for follow-up functional characterization, and the latter enabling us to test for a range of specific differential expression patterns, incorporating information from all four treatment groups. Moreover, by combining our ‘social RNA-Seq’ expression data with data on the anatomical location of transcript expression from a previously published ‘positional RNA-Seq’ study (Arbore *et al.* 2015), we were also able to investigate differential expression of putatively tissue-specific transcripts.

Differential expression (DE) analyses were first performed using *DESeq* version 1.8.3 (Anders & Huber 2010), implemented in Bioconductor v2.10 in R v2.15.1. Prior to analysis, all count data was rounded to the nearest integer value. After specifying the experimental design and estimating dispersion parameters based on the whole dataset, gene expression was compared for ca. 72,000 genes (i.e. all genes with count > 0 for at least one of the eight samples), contrasting expression in the four replicates of the *isolated* treatment with that in the four replicates of the *octets* treatment. The test involves use of a negative binomial model to assess each transcript (Anders & Huber 2010), followed by a Benjamini-Hochberg correction to control for multiple testing, adopting a false discovery rate (FDR) of 0.1.

We also explored the effect of two additional steps on the analysis: (i) adopting a more stringent ($=0.05$) FDR criterion and (ii) pre-filtering to remove a fixed percentage (either 20 or 40%; see (Anders & Huber 2010)) of transcripts with the lowest overall expression level. The former approach is more conservative, but runs the risk of generating false negatives, which could be counterproductive in our attempt to comprehensively identify differentially expressed candidates, especially given that we test for differences based on only 8 biological replicates; the latter approach has the advantage of giving us more power to detect (and thereby increases the yield of) differentially expressed candidates among those transcripts that remain (in total, 9,070 are called as DE with 40% pre-filtering, compared to 7,584 with no filtering), but might filter out low abundance transcripts that nevertheless differ consistently in expression between treatments. We therefore report in the main results the most inclusive approach as described above, but for completeness report also these additional analyses in Suppl. Table S2. The qualitative conclusions remain unchanged.

Since analyses using *DESeq* can only be performed on a pairwise basis, we next sought to estimate differential expression parameters using the program *baySeq* (Hardcastle & Kelly 2010), so that information from all 16 biological replicates could be incorporated into a single analysis, to thereby test for specific differential expression scenarios. *BaySeq* uses an empirical Bayesian framework to calculate posterior probabilities that each transcript in the dataset belongs to a certain ‘class’, where each class defines a particular pattern of expression. We defined eight classes: seven biologically plausible differential expression patterns (DE_A – DE_G) plus one in which there was no differential expression across all four treatment groups (NDE):

```
DEA = (octets, pairs), (joined, isolated)
DEB = (octets), (pairs), (joined), (isolated)
DEC = (octets), (pairs), (joined, isolated)
DED = (octets), (pairs, joined, isolated)
DEE = (octets, pairs, joined), (isolated)
DEF = (octets), (pairs, joined), (isolated)
DEG = (octets, pairs), (joined), (isolated)
NDE = (octets, pairs, joined, isolated)
```

where treatments contained within the same brackets do not differ in expression level. The program returns estimates of the proportion of transcripts belonging to each class, together with posterior probabilities for each transcript for each class. We then summed the relevant estimated proportion of transcripts for relevant DE classes to address three specific research questions: (1) what proportion of transcripts are differentially expressed between *isolated* and the two largest mating group size treatments of *pairs* and *octets* (‘not mating vs. mating’ transcripts, i.e. DE_A + DE_E + DE_G); (2) what proportion of transcripts are differentially

expressed between *pairs* and *octets* ('mating group size' transcripts, i.e. $DE_B + DE_C + DE_D + DE_F$); and (3) what proportion of transcripts are differentially expressed between *isolated* and *joined* worms ('early response' to mating transcripts, i.e. $DE_B + DE_E + DE_F + DE_G$)? Although the *baySeq* analysis was primarily implemented to estimate these broad scale patterns, the sum of posterior probabilities for different DE classes can also be used to judge the evidence for differential expression of specific transcripts. Moreover, where one particular DE class by itself exceeds a posterior probability of 0.95, we would interpret this as clear evidence for that specific pattern of expression.

Finally, to generate tissue-specific candidates for functional characterization, we cross-matched the differentially expressed candidates generated by the pairwise *DESeq* analysis to an existing dataset reporting positional patterns of gene expression in *M. lignano* (Arbore *et al.* 2015). Briefly, Arbore *et al.* generated tissue-specific candidates by examining gene expression in four different body regions of the worm: the head region (containing the rostrum, eyes, brain and pharynx with associated glands), the testis region (in which the space on both sides of the gut is primarily occupied by the paired testes), the ovary region (in which the space on both sides of the gut is primarily occupied by the paired ovaries) and the tail region (containing developing eggs, the female and male genitalia – including the seminal fluid-producing prostate gland cells – and the tail plate with its associated adhesive organs; see Fig. 4A for a morphological overview). By cutting worms into fragments corresponding to the boundaries between these regions, they generated four RNA pools for RNA-Seq containing either (A) only the head region, (B) the head and testis regions, (C) the head, testis and ovary regions, and (D) the head, testis, ovary and tail regions (i.e., whole worms). Using a differential expression criterion of a \log_2 -expression change >2 , this identified more than 4,000 transcripts changing in expression between adjacent samples – i.e. B vs. A, C vs. B, or D vs. C – indicating an enrichment for expression in the testis, ovary, or tail regions, respectively, and making these transcripts good candidates for having the

corresponding tissue-specific expression (which was validated by *in situ* hybridization in some cases). All transcripts were assigned to positional classes according to the expression pattern for these three fragment comparisons, meaning putative testis-specific candidates were designated as class [+ ,O,O], ovary-specific candidates as [O,+ ,O] and tail-specific candidates as [O,O,+]. Note that members of a further class of transcripts that increased in expression in both the B vs. A and C vs. B sample comparisons, i.e. class [+ ,+ ,O], were also interpreted as ovary-specific candidates owing to the fact that there was likely some contamination of ovary material in the testis (B) fragments (Arbore *et al.* 2015). This classification is now further supported by the highly similar expression responses to different social group sizes of classes [O,+ ,O] and [+ ,+ ,O] in our study (see below), and we therefore also interpret both classes as likely ovary-specific candidates. By far the largest class of transcripts were those that did not substantially differ between the different body regions, i.e. class [O,O,O]. Following Arbore *et al.*, we here term these ‘non-tissue specific’, but note that this simply means that we do not yet have any evidence for tissue-specific expression, rather than necessarily implying that these transcripts are ubiquitously expressed. A number of additional classes were also defined by Arbore *et al.*, but collectively the above five classes account for >99% of all transcripts.

***In situ* hybridization screen**

Whole-mount *in situ* hybridization (ISH) was performed for a total of 97 transcripts (using the primers specified in Suppl. File S2) according to standard protocols in *M. lignano* (Lengerer *et al.* 2014).

We attempted to investigate patterns of expression for a set of 15 transcripts previously identified as testis-specific candidates, haphazardly sampled to include both transcripts that are and are not differentially expressed in different social environments, and to include a

range of overall expression levels, with the qualifying criteria that the transcript had to be represented by at least ca. 100 reads in both the 'positional' (Arbore *et al.* 2015) and 'social' (this study) RNA-Seq datasets (because weakly expressed transcripts are difficult to assess with ISH), as well as that the transcript length had to be >300 base pairs (to facilitate ISH probe specificity). We similarly sampled 19 transcripts from the ovary-specific classes, as well as 63 transcripts from the non-specific class. These latter transcripts were included to begin to probe the potential sites of expression of this large class of transcripts and to ascertain whether expression in particular organs or tissue types appears to be over-represented.

In each case, primers were designed with Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) and a T7 promoter region was added at the 5' end of the reverse primers. Template DNA was produced using standard PCR reactions. In case for a particular transcript the PCR failed, it was repeated at least once using a different annealing temperature, and in most cases an additional time with a different batch of cDNA. To synthesize single stranded digoxigenin-labeled RNA probes, T7 polymerase (Promega) and DIG labelling mix (Roche) were used. Anti-digoxigenin-AP Fab fragments (Roche) were diluted 1:2000 and the signal was developed using the NBT/BCIP system (Roche) at 37 °C. Specimens were mounted in Mowiol and images were taken using a Leica DM5000 microscope.

RNAi screen

RNAi was performed for 12 putative testis- or ovary-specific, differentially expressed candidates (MLRNA110815_14701, MLRNA110815_21744, MLRNA110815_24606, MLRNA110815_1214.1, MLRNA110815_13829.2, MLRNA110815_3206.2, MLRNA110815_54, MLRNA110815_10574, MLRNA110815_1085, MLRNA110815_35903,

MLRNA110815_10265 and MLRNA110815_26815.1 – see Results for further explanation why these particular transcripts were selected), plus a positive control (macif1 (Lengerer *et al.* 2014)) and a negative control (no dsRNA), using standard protocols and a new batch of experimental subjects (prepared in a similar way as in the initial group size experiment).

Briefly, double-stranded RNA (dsRNA) probe was generated by an *in vitro* transcription system using primer pairs with SP6 and T7 promoter regions (T7 and SP6 Ribomax™ large scale RNA kit, Promega). DsRNA was diluted in artificial sea water (ASW) to a final concentration of 15 ng/μl. The solution was supplemented with algae and with antibiotics. Streptomycin, Kanamycin, and Ampicillin (50 μg/ml) were alternated every third day to prevent the selection of resistant bacterial strains. Treatments started from one week post-hatching and were maintained for three weeks. For each transcript, 30 animals were kept in embryo glass dishes (in 400μl of solution per dish). Every 24 hours, animals were transferred to a clean embryo glass dish and the dsRNA solution was changed. Throughout the whole experiment, animals were fed *ad libitum* and were maintained under normal culture conditions. At the end of the experiment, the efficacy of the RNAi knockdown was verified by performing ISH. The worms were then morphologically assessed for phenotypes under the microscope to assess testes area and ovary area (measured as described above), plus body area (at 40x magnification), egg number (counted under the microscope during imaging) and received sperm status (assessed by examining the antrum at 400x magnification for the presence or absence of sperm, which was sometimes inconclusive – and recorded as a third level of ‘unknown’ – since the presence of eggs in the antrum can make this difficult to discern). For body area, testes area, ovary area and egg number, the significance of treatment effects for each transcript, relative to the negative control, was assessed using ANOVA, adopting Dunnett’s method for comparing multiple means to a single control mean. For received sperm status, significance was assessed by means of 3x2 Fisher Exact Tests (Freeman-Halton extension) with a Bonferroni correction to the

significance threshold ($\alpha = 0.05/12 = 0.004$) to account for the multiple treatment comparisons to the single negative control. We note that for this initial, prospective screen, all worms receiving the same treatment were housed in the same embryo dish during the treatment phase. The specific results obtained should thus be interpreted with due caution, pending confirmation with a fully replicated experiment.

Results

Worms adjust their sex allocation to the prevailing social conditions

As predicted, group size had a highly significant effect on our proxy for sex allocation (one-way ANOVA, $F_{3,145} = 61.75$, $P < 0.0001$, Fig. 1): worms raised in the *isolated* treatment had the most female-biased sex allocation and worms raised in *octets* had the most male-biased sex allocation, with worms raised in *pairs* being intermediate. The fourth treatment group, termed *joined*, involved worms being raised initially as per the *isolated* treatment group, but then paired with another such worm from the same treatment for 24 h prior to the end of the experiment. In *M. lignano*, this should be long enough to ensure multiple matings (Schärer *et al.* 2004a), but not long enough for worms to adjust significantly their morphological sex allocation (Brauer *et al.* 2007) (as is also evident from the greatly overlapping SEs between *isolated* and *joined* worms in Fig. 1). The *joined* treatment group was included to identify putative ‘early response’ genes that change in expression upon commencement of mating (McGraw *et al.* 2004; 2008) (see ‘Transcripts affected by mating status’ below).

The socially sensitive transcriptome of M. lignano

Having shown that our experimental worms responded to their prevailing social environment by altering their sex allocation in agreement with our expectations, we next investigated the transcriptional basis of this response. Beginning with the pairwise

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comparison of the two most extreme treatment groups—and for now ignoring positional expression data, which is explored in the next section—our global estimate from comparing the *octet* and *isolated* treatments using *DESeq* is that 9.9% of the 76,437 investigated transcripts are differentially expressed, generating over 7,500 candidate transcripts that putatively differ in expression according to the social environment: specifically, 4,503 and 3,082 transcripts exhibit significantly higher or lower expression in *octets*, respectively (Suppl. File S3).

To illustrate some of this plasticity, expression patterns for the top 100 plastic candidates (based on statistical support for differential expression, i.e. adjusted p-values) are depicted in Fig. 2, alongside functional annotations extracted from the most recent genome-guided *M. lignano* transcriptome assembly (Wudarski *et al.* 2017). Note that this means the annotations come from a more recent transcriptome assembly than the one we employed here – details for inter-converting between these assemblies are provided in Suppl. Files S3 and S4, and the complete annotations used to produce Fig. 2 are provided in Suppl. File S5. Also note that in illustrating (where possible) the functional annotations of the top 100 plastic candidates, we did not restrict ourselves to only annotated genes, because one important pattern that emerges from this analysis is that many plastic genes remain unannotated, likely reflecting the phylogenetically distant position of *M. lignano* from some of the main organisms studied to date.

By initially focusing on just the *octet* and *isolated* treatments, and ignoring the intermediate treatments, we implicitly assumed that these two groups represent two extremes of a sex allocation continuum. This assumption is actually justified by subsequent analyses presented below (see ‘Genomic reaction norms for gametogenesis’), but to remedy the limitation we next performed *baySeq* analyses that allowed us to incorporate more complex differential

Accepted Article

expression patterns from all four treatment groups, and thereby test specific hypotheses about the nature of expression differences. Having estimated the proportion of transcripts in the whole transcriptome belonging to the range of different DE classes described above (Fig. 3A), we summed these estimated proportions for relevant DE classes to address specific research questions (Fig. 3B-D). First, we asked what proportion of transcripts are differentially expressed between *isolated* and the two largest mating group size treatments of *pairs* and *octets* (i.e. 'not mating vs. mating' transcripts, Fig. 3B), and second, what proportion of transcripts are differentially expressed between *pairs* and *octets* (i.e. 'mating group size' transcripts, Fig. 3C)? (Note that a third comparison – contrasting *isolated* and *joined* treatment groups – is covered in the 'Transcripts affected by mating status' section below.) In all cases, this analysis did not primarily aim to identify specific differentially expressed transcripts *per se* (a statistically more difficult task, Hardcastle & Kelly 2010), but rather to estimate the proportion of transcripts belonging to each DE class. We note, however, that there was a strong overlap between the *baySeq* analysis and the pairwise *DESeq* comparison described above with respect to the transcripts that these analyses identified as differentially expressed, with 92.4% of differentially expressed candidates that could be identified by *baySeq* also identified as such by *DESeq* (data not shown).

The *baySeq* analysis confirms that a substantial portion of the *M. lignano* transcriptome – around one fifth of all transcripts – is socially sensitive in its expression. Specifically, we estimated that 10.63% of the transcriptome (ca. 7950 transcripts) belong to a DE class in which transcript expression in *isolated* worms differs from that in *pairs* and *octets* (Fig. 3B), with an additional 8.39% (ca. 6250 transcripts) differing in expression between *pairs* and *octets* (Fig. 3C).

Defining tissue-specific transcriptional responses to altered group size

We next sought to investigate gene expression responses for transcripts predominantly expressed in particular body regions and thus having putatively tissue- and sex-specific expression, based on the positional separation of the male and female sex functions in the worm's anatomy (Fig. 4A). When splitting our pairwise *DESeq* dataset according to the positional classifications identified in an earlier study (see Materials and Methods), by far the largest number of DE transcripts identified here belong to the non-tissue specific class [0,0,0] that did not greatly differ in expression between different body regions, with approximately equal numbers of transcripts having higher ($n = 3302$) and lower ($n = 2826$) expression in larger groups (Fig. 4B). However, there are a very large number of transcripts in this class, and so this socially-sensitive fraction still only represents <10% of all non-specific transcripts. By contrast, typically >30% of transcripts were differentially expressed for the putatively tissue-specific classes that had previously been shown to exhibit higher expression in particular body regions (see below). Thus, tissue-specific candidates appear at least three times as likely to be socially-sensitive in their expression, as might be expected given that the putative tissue specificity refers to those tissues most likely to be involved in a sex allocation response to group size. We also emphasize again that just because a transcript belongs to class [0,0,0], this does not necessarily mean that it is ubiquitously expressed, only that – based on the data so far available – we have so far found no evidence for a specific expression pattern. For completeness, a summary of differential expression according to the remaining 17 positional classes defined in Arbore *et al.* (2015) is given in Suppl. Table S3, but in the next sections, we focus solely on these three main putatively tissue-specific classes, i.e. transcripts that represent strong candidates for being either testis-specific (class [+ ,0,0]), ovary-specific (classes [0,+ ,0] and [+ ,+ ,0]) or tail-specific (class [0,0,+]). Recall, however, that in each case, the assignment as putatively tissue-specific stems solely from the significant increase in expression of the respective transcript in the body region containing the male or female tissue of interest, and so for the majority of transcripts identified as being potentially involved in gametogenesis, we have not yet confirmed whether the transcripts are

truly testis- or ovary-specific (but see '*In situ* hybridization screen for tissue-specificity of differentially expressed candidates' below).

Transcriptomic reaction norms for gametogenesis

As predicted by sex allocation theory and expected based on the above-reported morphological sex allocation response, sex allocation plasticity is also clearly visible from a molecular perspective: 1053 (out of 3360, i.e. ca. 31%) of testis-specific candidate transcripts belonging to the class [+ ,0,0] exhibited evidence for differential expression, and the vast majority of these ($1033/1053 = 98\%$) had higher expression in larger groups (Fig. 4C). 125 (out of 323, i.e. ca. 39%) of ovary-specific candidate transcripts belonging to the class [0,+ ,0] and 80 (out of 127, i.e. ca. 63%) of ovary-specific candidate transcripts belonging to the class [+ ,+ ,0] exhibited evidence for differential expression (i.e., ca. 46% overall), but the vast majority of these ($200/205 = 98\%$) exhibit lower expression in larger groups (Fig. 4D and 4E). These expression responses by putatively testis- and ovary-specific candidates presumably to some extent directly represent the switch away from oogenesis and towards spermatogenesis in larger groups, i.e. their expression pattern is at least partially accounted for by the relative shift in the amount of testicular and ovarian tissue. Nevertheless, it seems unlikely that the expression changes we have documented can be accounted for purely by changes in gonad size. Taking the testis as an example – arguably the organ we know most about in *M. lignano* – the two-dimensional area we measure in squeezed worms probably quite closely reflects the changes in testis volume occurring between treatments. Comparing *isolated* and *octet* worms, we found on average testis areas of 9616 mm² and 14981 mm², respectively, implying a 1.56x increase in testis volume in octets. So, if the transcript expression changes were purely in line with this morphological response, we would expect all transcripts to change in expression by ca. 1.56x. This is clearly not the case, since we observed substantial heterogeneity in the expression changes (with fold-changes ranging

from 0.02 to 7.08, and 94% of differentially expressed transcripts exhibiting fold-changes in excess of 1.56x), with an average change somewhat higher than expected based on morphology alone (mean fold-change 2.14x, median 2.02x). We also identified 20 transcripts that had significantly lower expression in octets, i.e. the opposite trend to that predicted by the morphological response. These ‘exceptional’ transcripts (plus the corresponding five putatively ovary-specific candidates that had higher expression in octets) might represent particularly interesting transcripts for functional characterization in the context of (negative) regulation of spermatogenesis and oogenesis.

The contrasting responses for male and female allocation can also be visualized as tissue-specific reaction norms for all differentially expressed testis- and ovary-specific candidates (Fig. 5A-C; tail-specific candidates are discussed in the next section), which clearly demonstrate a quantitative shift in gene expression that is in close qualitative agreement with the morphological response, i.e. the greatest difference in expression levels is between *isolated* and *octet* worms, with intermediate expression levels in *pairs*. For the 1,053 putative testis-specific candidates the prevailing trend is clearly positive with group size, with an approximately two-fold (i.e. one \log_2 unit) average increase in expression from *isolated* to *octets* (Fig. 5A). For the 125 putative ovary-specific candidates in class [0,+,0] there is a clear and complementary negative trend of approximately equal magnitude (Fig. 5B), as there is also for the 80 putative ovary-specific candidates in class [+,+,0] (Fig. 5C). Together with the disproportionately large number of sex-specific transcripts in the pool of socially sensitive genes, this graded response across successive treatment groups argues strongly against one possible interpretation of our data, namely that the treatment of social isolation might induce a generalized stress response resulting in widespread downregulation of gene expression (as has sometimes been observed in other systems with more obvious stressors, e.g. (Causton *et al.* 2001; Levine *et al.* 2011; Yampolsky *et al.* 2014)). Instead, the tissue-specific transcriptional responses we observe are in line with the graded

morphological sex allocation response found in this and previous experiments (Schärer & Janicke 2009; Janicke *et al.* 2013).

Novel aspects of male allocation

In addition to characterizing the quantitative changes in gene expression that occur in the gonads as a result of a shift in investment between spermatogenesis and oogenesis, our study likely also reveals novel aspects of sex allocation in *M. lignano*. Notably, a large proportion of the transcripts with putative tail-limited expression ($150/366 = \text{ca. } 41\%$) exhibits evidence of marked differential expression according to the social context (Fig. 4F, Fig. 5D), with an approximate seven-fold difference in expression between the two most contrasting social groups (cf. the above-mentioned two-fold difference for testis- and ovary-specific candidates). The most obvious morphological features located in the tail of the worms that might exhibit such an expression pattern are the prostate gland cells, which produce seminal fluid substances added to sperm in the ejaculate (Ladurner *et al.* 2005b; a; Vizoso *et al.* 2010; Weber *et al.* 2018). These 150 transcripts, and especially the 140 that increase in expression with group size, are thus strong candidates for prostate-specific genes involved in seminal fluid production, a previously unquantified component of male allocation. This conclusion is based on the similar reaction norms for most of these transcripts compared to those involved in the other major aspect of male allocation (i.e. testis-specific genes; Fig. 5A), as well as by the striking degree of plasticity in their expression. Importantly, this functional assignment has in many cases now been confirmed by *in situ* hybridization data: prostate-specific expression was already reported for MLRNA110815_22046, MLRNA110815_80.4 and MLRNA110815_9549.4 (Arbore *et al.* 2015), and a comprehensive screen of nearly all remaining transcripts, conducted as part of a project on seminal fluid diversity and function in *M. lignano*, has revealed that ca. two-thirds indeed exhibit prostate-specific expression, 76 of which are expressed exclusively in prostate gland cells (Weber *et al.* 2018).

Transcripts affected by mating status

Finally, to examine putative ‘early response’ genes whose expression changes following the onset of mating, we contrasted *isolated* worms to the remaining three treatment groups. The *baySeq* analysis suggests 0.37% of all transcripts (i.e. around 280 transcripts) belong to such a DE class (Fig. 3D). However, although this estimate suggests that there are a considerable number of transcripts that are differentially expressed according to mating status, we had relatively little power to identify them with our experimental design. In fact, only three individual transcripts in the *baySeq* analysis passed the posterior probability threshold of 0.95 allowing them to be unambiguously assigned as differentially expressed candidates. MLRNA110815_2487.2 (which exhibits significant homology to human gene NRDE2) and MLRNA110815_34533.1 (which exhibits significant homology to the testis-enriched human gene NCAPH, non-SMC condensin I complex, subunit H) (Uhlén *et al.* 2015) were both more highly expressed in *isolated* worms compared to the other treatments (though the very low overall expression level of the former urges caution in this conclusion) whereas MLRNA110815_892.8 (no significant human homology) exhibited a ca. two-fold lower expression in *isolated* worms compared to the other treatments.

In situ hybridization screen for tissue-specificity of differentially expressed candidates

To begin to probe the potential functions of the identified differentially expressed transcripts and to validate our interpretation of likely tissue-specific plasticity in gametogenesis genes, we performed an *in situ* hybridization (ISH) characterization of the sites of expression for a subset of transcripts sampled from across the distributions of putatively testis-, ovary- and non-specific classes.

Beginning with the testis, we attempted to investigate patterns of expression for a set of 15 transcripts previously identified as testis-specific candidates (Arbore *et al.* 2015). While five transcripts could not be investigated due to the inability to design suitable primers or due to failed PCRs, for the remaining ten transcripts we could predict that the eight differentially expressed candidates would show testis-specific expression. By contrast, the two other remaining candidates that did not show socially-sensitive differential expression are presumably less likely to be testis-specific in their expression. The ISH data support these predictions, with all but one fitting the expected pattern (Fig. 6A-K).

Similarly, for the ovary we attempted to investigate 19 transcripts identified as ovary-specific candidates (n=7 from class [o,+,o] and n=12 from class [+,+,o]), for which PCR failed in six cases, leaving 13 candidates. We could predict that ten of these would be more likely to be truly ovary-specific (because of their differential expression in *isolated* and *octet* worms) whereas the remaining three would be less likely to be ovary-specific. Again, the ISH patterns support this contention, with seven out of the ten transcripts expected to be ovary-specific indeed exhibiting ovary-limited expression (and the three others also being expressed in the ovary, but further exhibiting weak staining in the testis or at the testis periphery). For the three candidates that are not socially sensitive in their expression, one is expressed in the ovary and in developing eggs and two are expressed in both gonads (Fig. 6L-Y).

Our differential expression calls can also be compared to previously reported ISH patterns (Arbore *et al.* 2015), and these too largely support our main conclusions. Taking first the transcripts expressed in the testis region, Arbore *et al.* (2015) determined that four out of six candidates (based on positional RNA-Seq data) were actually testis-limited (based on ISH patterns). Of these four transcripts, three show strong testis-specific staining and are (as

might be predicted) also socially sensitive based on our social RNA-Seq data (MLRNA110815_7008, MLRNA110815_9973.1, MLRNA110815_6628.2). A fourth transcript, MLRNA110815_3228, was found to exhibit a weakly testis-specific signal in ISH experiments and shows no evidence of socially sensitive differential expression in our study. Of the remaining two testis region candidates found by Arbore et al. (2015) not to be testis-limited in their expression pattern (but rather expressed in the gut epithelium), one (MLRNA110815_10311.2) is differentially expressed in our analysis and the other (MLRNA110815_9262) is not. For the ovary region, six out of eight putatively ovary-specific candidates investigated indeed had ovary/developing egg-limited expression (MLRNA110815_16738, MLRNA110815_1618.1, MLRNA110815_7725.2, MLRNA110815_7498, MLRNA110815_2640 and MLRNA110815_4558) and all but one (MLRNA110815_7498) show evidence of the predicted differential expression in our dataset. For the two remaining transcripts for which no ISH pattern was obtained, one exhibits differential expression in the same direction (MLRNA110815_6266) and the other does not (MLRNA110815_12337.1).

Finally, as described above, by far the biggest class of transcripts found to exhibit evidence of socially-sensitive gene expression were those that did not show a clear pattern of differential expression in the earlier positional RNA-Seq study (Arbore *et al.* 2015), i.e. those belonging to class [0,0,0]. To begin to probe the potential expression patterns of this very large number of candidates, we selected a subset of 63 transcripts for exploratory characterization by ISH, revealing a wide range of expression patterns, including many even within this class that exhibited specific expression in reproductive organs (Suppl. Fig. S1).

RNAi confirms roles for differentially expressed candidates in reproductive pathways

Although a comprehensive functional picture is beyond the scope of this study and must await the outcome of future follow-up experiments, we performed a small-scale prospective RNAi screen of putative testis- and ovary-specific transcripts to confirm that the many differentially expressed candidates we have identified are indeed likely to play roles in gametogenesis pathways, selecting 12 transcripts from among the differentially expressed testis- and ovary-specific candidates. Specifically, most transcripts exhibited the prevailing pattern in the testis (up in octets: MLRNA110815_14701, MLRNA110815_21744, MLRNA110815_24606) or ovary (down in octets: MLRNA110815_1214.1, MLRNA110815_13829.2, MLRNA110815_3206.2, MLRNA110815_54, MLRNA110815_10574, MLRNA110815_1085, MLRNA110815_35903), with the remaining two exhibiting the reverse pattern (testis transcripts down in octets: MLRNA110815_10265, MLRNA110815_26815.1). As a negative control, we included an additional treatment group that was treated identically to the RNAi treatments except that no dsRNA probe was added to the relevant dish. As a positive control, we also performed RNAi on another tail-specific transcript, *macif1*, that functions in the anchor cells of the adhesive system, rather than in the reproductive system, and for which a clear RNAi phenotype—the inability to adhere to a substrate—was expected (Lengerer *et al.* 2014).

We verified whether knockdown was successful by conducting a subsequent ISH experiment (using the same methods as described above) on the treated and control worms: 7 out of 12 transcripts were successfully knocked down, as determined by the absence of staining – or in the case of RNA815_26815.1 and RNA815_54 reduced staining implying partial knockdown – on ISH (not shown). In total we observed 9 cases where one of the five measured phenotypic traits – body area (Fig. 7A), testes area (Fig. 7B), ovary area (Fig. 7C), total egg number (Fig. 7D) or received sperm score (Fig. 7E) – differed between a specific RNAi treatment and the control. For body area, MLRNA110815_14701 knockdown worms were

significantly smaller than controls (ANOVA, Dunnett's posthoc test for multiple-to-one comparisons: $|\text{diff}| - \text{LSD} = 7239$, $P = 0.009$) and MLR_{NA110815_21744} knockdown worms were significantly larger than controls ($|\text{diff}| - \text{LSD} = 6997$, $P = 0.0095$). Both MLR_{NA110815_14701} ($|\text{diff}| - \text{LSD} = 7262$, $P < 0.0001$) and MLR_{NA110815_1214.1} ($|\text{diff}| - \text{LSD} = 249$, $P = 0.02$) had significantly larger testes than controls. Both MLR_{NA110815_54} ($|\text{diff}| - \text{LSD} = 250$, $P = 0.007$) and MLR_{NA110815_10574} ($|\text{diff}| - \text{LSD} = 389.3$, $P = 0.002$) had significantly larger ovaries than controls. MLR_{NA110815_1214.1} knockdown worms contained more developing eggs than controls ($|\text{diff}| - \text{LSD} = 0.054$, $P = 0.03$). Finally, for both MLR_{NA110815_14701} (Fisher Exact Test: $P = 0.0004$) and MLR_{NA110815_1214.1} ($P = 0.00002$), we found a smaller proportion of worms with received sperm in their antrum. 8 out of these 9 effects occurred among the seven transcripts where we could confirm that the knockdown was successful, strongly suggesting that the phenotypes we observed were a result of the specific knockdown (the exception was the larger than expected body size observed for the MLR_{NA110815_21744} RNAi).

To illustrate and expand on these observations with respect to specific transcripts, the MLR_{NA110815_14701} RNAi phenotype (which we had selected based on testis-specific expression, Fig. 6C) involved smaller body size (Fig. 7F), markedly larger testes size apparently containing an accumulation of sperm (Fig. 7G), and very few worms observed with received sperm; mature sperm released from the testis exhibited the same general morphology as the sperm in control worms (an anterior feeler, a posterior shaft, a pair of lateral bristles and a terminal brush; Ladurner *et al.* 2005b; Vizoso *et al.* 2010), but in contrast to controls, a high number of sperm showed a marked bend in the shaft (Fig. 7H) and most were partially or completely immobile. For MLR_{NA110815_1214.1} (selected on the basis of specific expression in ovaries and developing eggs, Fig. 3W), knockdown resulted in a complete absence of worms observed with received sperm, moderately larger testes and more eggs (Fig. 7I). As can be seen in Figs. 7J-L, the MLR_{NA110815_10574} and

MLRNA110815_54 knockdowns – both of which were selected based on ovary-specific expression, with MLRNA110815_54 also being expressed in developing eggs (Fig. 3S,Y) – had markedly larger ovaries compared to control worms. As expected, control worms exhibited the usual non-adhesive phenotype associated with *macif1* knockdown (Lengerer *et al.* 2014).

Collectively, these RNAi results correspond well to the expected expression patterns identified through our RNA-Seq and ISH experiments, and clearly demonstrate that our screen has identified a plastic subset of the *M. lignano* transcriptome that includes transcripts with specific functions in reproductive tissues. They also illustrate our ability to manipulate expression of these transcripts and thereby test their functions experimentally.

Discussion

Our results demonstrate that a large proportion of the *M. lignano* transcriptome is socially-sensitive in its expression, especially with respect to the subset of the transcriptome expressed uniquely in specific reproductive tissues. This is precisely as expected if the optimal gene expression pattern depends upon the particular ecological context in which an organism finds itself. In this case, the response is based on interactions with conspecifics affecting the overall level of sperm competition experienced and thus affecting the optimal balance of reproductive investment into the male and female sex functions, i.e. sex allocation. In the following, we first discuss the general finding that social conditions strongly impact upon gene expression patterns, then explore some of the specific insights we have gained with respect to sex allocation in hermaphrodites and compare these results to patterns of sex-specific transcript expression in species with separate sexes.

Global patterns of gene expression plasticity

To put the global patterns of plastic gene expression in context, we can compare our results to previous studies that have investigated genome-wide responses to social environmental conditions, predominantly in separate-sexed taxa. For example, Zhou et al. (2012) performed a microarray-based study of how expression varies in *D. melanogaster*, based on measuring gene expression in whole flies raised in one of 20 different treatment groups designed to manipulate different aspects of the environment. They found that overall around 8% of the transcriptome (1249/14400 transcripts investigated) is plastic in its expression. The majority of these treatments sought to manipulate physical, nutritional or chemical aspects of the environment, but around 0.25-1.34% of the genome was differentially expressed in different social environments (larval crowding, adult crowding or mating status). Note, however, that this figure does not include the class of genes (1.4%) that exhibit a significant sex \times treatment interaction. The social environment can also play a major role in triggering wholesale changes in gene expression underlying alternative morphs in taxa with alternative male reproductive tactics (Fraser *et al.* 2014), or in social insects with alternative castes (Sumner *et al.* 2006). The changes in gene expression that occur in the gonads of fish following changes in social rank or adopting different morphs are also particularly interesting in this context, as it may often pay for different males to differentially invest in spermatogenesis (Todd *et al.* 2018). And even more radical alternations in gene expression occur during sex change in sequential hermaphrodites (Liu *et al.* 2015). Finally, it is increasingly recognized that social factors are a significant influence on human gene expression (Slavich & Cole 2013).

Sex function-specific effects

Our data provide strong evidence that altering the social environment disproportionately impacts upon the expression of genes that are likely to differentially affect one or the other

sex function in *M. lignano* (Fig. 3). This is clear from the patterns of differential expression found for the putative testis-, ovary- and tail-specific classes, as well as the dominance of these classes among the 100 most plastic transcripts illustrated in Fig. 2: fully half of these are tail-specific candidates (90% of which have confirmed prostate-specific expression, and 76% exhibit prostate-limited expression; Weber *et al.* 2018)), and a further 15 are either testis- or ovary-specific candidates. Moreover, 38 of the top 100 plastic candidates did not exhibit significant homology to humans or three model organisms (i.e. the fruit fly *D. melanogaster*, the nematode *C. elegans* or the triclad flatworm *S. mediterranea*), and a further two only to *S. mediterranea*. This is consistent with the idea that many plastic transcripts are likely to be phylogenetically restricted or lineage-specific, as might often be expected for genes with sex-specific functions (Begun & Lindfors 2005; Begun *et al.* 2007; Haerty *et al.* 2007). Among those that did match to other taxa, there are also some hints at potential reproductive functions. For example, one putatively gonad-specific transcript (MLRNA110815_3206.3) matched to the SPATIAL protein family linked to spermatid differentiation in mouse (Saade *et al.* 2007), and several putatively tail-specific candidates belong to the metallo-peptidase family M12B reprotolysin-like, which includes the sperm surface membrane protein ADAM2 implicated in mammalian sperm-egg and/or sperm-female reproductive tract interactions (Cho 2012; Choi *et al.* 2016). We now know that at least two of these (MLRNA110815_5875.1 and MLRNA110815_5404.3) exhibit highly similar expression patterns in both the testis (weakly) and prostate, although with additional expression also in the pharynx region (Weber *et al.* 2018), which is at least partially consistent with a conserved reproductive function.

Our results can be compared to the increasing number of studies that have used similar methods to document sex differences in gene expression in separate-sexed taxa, in order to identify the genes underlying sexual dimorphism and shaped by sexual selection and sexual antagonism (reviewed in Wilkinson *et al.* 2015; Grath & Parsch 2016). This is of course a

Accepted Article

somewhat different question, since in simultaneous hermaphrodites there is no possibility of true sexual dimorphism, and individuals instead face an optimisation challenge of balancing investment into the male and female sex functions so as to maximise their net fitness returns (Schärer *et al.* 2015; Schärer & Ramm 2016). Nevertheless, there are clear parallels. Recent studies in *D. melanogaster* found that 8% of the genome experiences sexually antagonistic selection when expressed in either males or females (Innocenti & Morrow 2010), though a much higher proportion of genes exhibit some degree of sex bias in their expression level (Ranz *et al.* 2003). Moreover, it has been suggested that the imposition of monogamy selects for reduced sexual dimorphism, ‘feminizing’ male gene expression patterns, potentially due to reduced sexual antagonism (Hollis *et al.* 2014). Similarly, in wild turkeys (*Meleagris gallopavo*), compared to dominant males the gene expression of subordinate males is ‘de-masculinized’ for male-biased genes and ‘feminized’ for female-biased genes, corresponding to their intermediate, sexually dimorphic phenotype (Pointer *et al.* 2013).

In this study, rather than considering fixed differences between individuals differing in sex or social status, we asked instead how the same (hermaphroditic) individual might plastically shift gene expression in different environments favouring more male- or more female-biased sex allocation. In both cases the subset of genes differing in expression between the two states can be thought of as being sexually antagonistic, in that their expression optimum differs between the sexes or sex functions (which in simultaneous hermaphrodites equates to antagonistic pleiotropy) (Abbott 2010; Jordan & Connallon 2014; Schärer *et al.* 2015). Finally, perhaps the most directly comparable study to ours in separate-sexed organisms – at least in terms of male investment – is a recent exploration of transcriptome-wide expression responses to different socio-sexual environments in *D. melanogaster* (Mohorianu *et al.* 2017). This study sought to measure gene expression responses by focal males after various time periods of exposure to rivals, to identify candidate genes implicated in the highly plastic responses of male flies to perceived changes

Accepted Article

in immediate sperm competition risk and intensity. They further split these responses according to two body regions, the head/thorax and abdomen, expected to correspond to responses occurring in the nervous and reproductive systems, respectively. Substantial initial changes in expression in the head/thorax region were observed, supporting the idea that males are responding to multiple sensory cues. By contrast, the abdomen region was overall much more stable in expression, which could imply that the plasticity observed in flies under these circumstances is primarily at the level of allocation rather than production of ejaculate components. Nevertheless, the authors noted that several seminal fluid (but not sperm) genes changed markedly in expression between treatments, although this was not always consistent between replicates. Our results clearly confirm and extend these findings, demonstrating that a sustained exposure to different sperm competition levels in *M. lignano* results in broad-scale and marked differences in expression of genes implicated in both aspects of ejaculate production, i.e. sperm and seminal fluid.

Another interesting observation is the preponderance of differentially expressed testis- over ovary-specific transcripts in our dataset. Although a similar proportion of transcripts are differentially expressed in these two tissues, the far greater number of testis-specific transcripts overall means that there are at least five-fold more socially-sensitive testis-specific candidate transcripts ($n \leq 1,053$) than ovary-specific transcripts ($n \leq 205$; i.e. $n \leq 125$ in class [0,+,0] plus $n \leq 80$ in class [+,+,0]). This apparent male-bias in the number of sex-specific genes appears to be a common phenomenon, with similar evidence found, for example, in *D. melanogaster* and *C. elegans* (Reinke *et al.* 2000; Chintapalli *et al.* 2007; but see Mank *et al.* 201) for an opposite trend in chickens; reviewed in Kaessmann 2010; Ramm & Schärer 2014; Ramm *et al.* 2014).

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Given our clear results for seminal fluid as well as putatively testis-specific genes, our study further emphasizes the likely importance of non-sperm aspects of male allocation. The function and evolutionary significance of seminal fluid-mediated fitness traits is increasingly well recognised in a range of taxa (Poiani 2006; Avila *et al.* 2011), stressing the importance of an integrative approach to male reproductive investment (Simmons & Fitzpatrick 2012). Moreover, the rapid evolution and divergent expression of seminal fluid proteins has been linked to inter-specific or inter-population variation in sperm competition levels (Dorus *et al.* 2004; Ramm *et al.* 2008; 2009; Goenaga *et al.* 2015; Hollis *et al.* 2016). Our results highlight the scope also for considerable intraspecific plasticity in seminal fluid expression (see also (Patlar *et al.* 2018)). This is, to our knowledge, the largest scale evidence of such plasticity reported to date (but see Hollis *et al.* 2016 for a similar evolutionary response), supporting studies in separate-sexed taxa indicating plasticity in the expression of seminal fluid genes driven by variation in the socio-sexual environment, which likely modulates the demand for seminal fluid. A small number of investigated seminal fluid genes and proteins exhibit such plasticity in *Drosophila* (Fedorka *et al.* 2011; Wigby *et al.* 2015) (but see Mohorianu *et al.* 2017 – discussed above – for evidence that seminal fluid expression responses can be highly variable), in mice, the relative composition of the seminal fluid proteome differs according to sperm competition risk (Ramm *et al.* 2015), and in the cricket *Teleogryllus oceanicus*, males adjust seminal fluid production in response to cues of both sperm competition risk and intensity (Simmons & Lovegrove 2017; Sloan *et al.* 2018). Our data now provide a rich repertoire of candidates for probing seminal fluid function in a simultaneously hermaphroditic model system, in which post-copulatory sexually selected processes, such as those mediated by seminal fluid, are expected to be especially important, and for which unique functional hypotheses about seminal fluid have been proposed (Charnov 1979; Michiels 1998; Michiels & Koene 2006; Marie-Orleach *et al.* 2013; Nakadera *et al.* 2014; Schärer *et al.* 2015).

In the context of male allocation, another class of genes that might repay further investigation is the small number of transcripts whose positional expression is high in both the testis region (i.e. B vs. A contrast) and the tail region (i.e. D vs. C contrast) (Arbore *et al.* 2015). Although, according to Arbore *et al.* (Arbore *et al.* 2015), there are only nine such transcripts in this [+0,+] class, our analyses indicate that fully eight of these are differentially expressed between *isolated* and *octet* worms (MLRNA110815_14112, MLRNA110815_18363.1, MLRNA110815_18363.2, MLRNA110815_2216.1, MLRNA110815_324.2, MLRNA110815_471.1, MLRNA110815_471.2 and MLRNA110815_5404.2, but not MLRNA110815_4414). One plausible interpretation of such a pattern could be that these transcripts are expressed only in male tissues, or possibly even that they exhibit sperm-limited expression, though to date we have no direct evidence on their specific sites of expression. Several also remain functionally unannotated based on (absent) homology to other taxa, but interestingly MLRNA110815_18363.1 and MLRNA110815_18363.2 exhibit homology to the human gene WSCD2, which is enriched in male accessory reproductive glands (prostate, seminal vesicle) and MLRNA110815_2216.1 exhibits homology to ABCA8, which is enriched in both male and female gonads (Uhlén *et al.* 2015).

Outlook and conclusions

Advances in sequencing technologies mean that the molecular basis of phenotypic plasticity can now be studied, defining “genomic reaction norms” for traits of ecological or evolutionary significance (Aubin-Horth & Renn 2009; Alvarez *et al.* 2015). We have shown that a substantial proportion of the *M. lignano* transcriptome is differentially expressed according to the prevailing social conditions, as worms shift their investment from a more female-biased sex allocation at small group size towards a more male-biased sex allocation at larger group size, in line with predictions from sex allocation theory (Charnov 1979; 1982).

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These data establish the key role played by the biotic environment in generating variation in gene expression in this species, and provide significant insights into how sex allocation plasticity is achieved. Of course, understanding the genes exhibiting altered expression in response to sex allocation plasticity represents a necessary but still insufficient first step towards a complete mechanistic understanding of the phenomenon. Nevertheless, we are now equipped with a comprehensive repertoire of candidate genes for functional characterization from our genome-wide scan, and have demonstrated that molecular tools such as ISH and RNAi are readily applicable in *M. lignano* (see also e.g. Sekii *et al.* 2009; 2013), enabling us to now test the functional and evolutionary significance of individual genes. Moreover, the genus *Macrostomum*, comprising >150 described species, represents an excellent model taxon for complementary genomic and transcriptomic analyses of the rapid divergence of sex allocation and sexually selected traits in hermaphrodites (Schärer *et al.* 2011). It will, for example, be fascinating to examine whether the striking pattern of more rapid evolution among sex-specific genes in species with separate sexes holds up also for sex-specific genes in this hermaphroditic animal taxon. There is some evidence in the hermaphroditic plant *Arabidopsis thaliana* suggesting that at least some male-specific genes, expressed in pollen and pollen tubes, indeed show a higher incidence of positive selection (based on patterns of intraspecific polymorphism and interspecific divergence from *A. lyrata*) (Gossmann *et al.* 2014).

For now, our results define the genes that are differentially expressed in environments favouring shifts in investment towards one sex function or the other in this model hermaphroditic animal, and thus begin to bridge organismal and molecular perspectives on phenotypic plasticity in sex allocation, a crucial life history trait.

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Data accessibility

Data associated with the study, including the MLRNA110815 transcriptome assembly, RNASeq data and derived read counts, has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE123594 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123594>). Raw data are also deposited in the Sequence Read Archive under accession number SRP174213 (<https://www.ncbi.nlm.nih.gov/sra?term=SRP174213>).

Author contributions

Study conception and experimental design: SAR, LS. Performed the main experiment: SAR. Performed the sex allocation assay: SAR, LS. Extracted RNA: RA. Mapped the sequenced reads: EB. Performed the ISH and RNAi screens: BL, RP, JW, AG and PL. Analysed the data and drafted the manuscript: SAR, LS. All authors contributed to manuscript revisions, and read and approved the final manuscript.

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Figure Captions

Figure 1. Sex allocation plasticity in *Macrostomum lignano* from a morphological perspective.

The investment into testes (Te, blue) and ovaries (Ov, orange) can be readily quantified in these transparent flatworms (as illustrated in the inset) and used to derive a proxy for sex allocation (as testis area / (testis area + ovary area)). We confirmed that sex allocation varies significantly with the group size treatment (see main text for details). This represents a by now well-established phenotypically plastic response that we here investigate further from a transcriptional landscape perspective.

Figure 2. Per replicate expression patterns and functional annotations for the top 100 plastic candidate transcripts differing in gene expression according to the social environment in *Macrostomum lignano*.

For each of the top 100 plastic candidates, we plot their expression patterns in the four replicate isolated treatments (blue) and four replicate octet environments (red), together with a summary of functional information including their positional expression patterns in *M. lignano* (Arbore et al. 2015), the presence (filled squares) or absence (open squares) of identified homologs in humans (Hm) and three model organisms (*Drosophila melanogaster*, Dm; *Caenorhabditis elegans*, Ce; *Schmidtea mediterranea*, Sm) as well as the identity of the top (lowest E-value) human homolog and top scoring Pfam assignment. For full functional annotations, see Suppl. File S5, and for similar functional annotations of all remaining transcripts please see those provided for the Mlig_RNA_3_7_DV1 transcriptome assembly (Wudarski et al. 2017, Grudniewska et al. 2018), in combination with Suppl. Tables S3 and S4, which provide corresponding transcript IDs for inter-converting between assemblies.

Figure 3. Global patterns of differential gene expression according to the social environment in *Macrostomum lignano*.

We began by incorporating information from all transcripts to gain a global impression of differential expression patterns in the *M. lignano* transcriptome. (A) Using *baySeq*, we estimated the likelihood that each transcript belonged to one of eight different differential expression classes (seven biologically plausible patterns in which at least one treatment group differed in expression level compared to the others, plus a ‘not differentially expressed’ class in which all four treatment groups did not differ from one another). We then summed posterior probabilities for all transcripts to estimate the total number of transcripts belonging to one of the seven different differential expression classes (ca. 19% of all transcripts, as depicted in the pie chart). Next, we examined the estimates from relevant classes, depicted separately below the pie chart and highlighting with dotted borders the specific type of differential expression each class represents: treatments surrounded by the same border do not differ from each other, whereas those surrounded by different borders do differ from one another. We used these estimates to address the proportion of the transcriptome corresponding to three specific biological hypotheses: (B) that a subset of the transcriptome is differentially expressed between *isolated* and grouped (*pair* and *octet*) worms, i.e. transcripts differentially expressed when ‘not mating vs. mating’; (C) that a subset of the transcriptome is differentially expressed between *pair* and *octet* worms, i.e. transcripts differentially expressed according to ‘mating group size’; and (D) that a subset of

the transcriptome is differentially expressed between *isolated* and *joined* worms, i.e. transcripts differentially expressed as an ‘early response’ when commencing mating. For each scenario (B-D), we summed all differential expression classes that fulfil the criterion stated in the scenario (as highlighted in blue and red) to arrive at the estimated total proportion of the transcriptome to which this scenario could apply (stated below).

Figure 4. The transcriptional landscape of sex allocation plasticity in *Macrostomum lignano*.

(A) Photomicrograph and line drawing of the model organism (adapted from (Schärer *et al.* 2007)), indicating key morphological features (total body length ca. 1.5mm). The antrum is the female sperm-receiving organ, the seminal vesicle is the male sperm storage organ and the stylet is the male copulatory organ. Owing to their transparency, previous transcriptomic data has been generated from worms cut into fragments corresponding to the boundaries between key reproductive traits (Arbore *et al.* 2015), thereby enabling us here to assign the 76,437 transcripts analysed as putative candidates expected to have either non-specific, testis-specific, ovary-specific or tail-specific expression, as indicated in the smaller inset line drawings of worms in subsequent panels. Differentially expressed candidates belonging to each of these positional classes are plotted separately in colour in the subsequent panels: (B) non-specific [0,0,0] (red), (C) testis-specific [+ ,0,0] (blue), (D) ovary-specific [0,+ ,0] (orange), (E) ovary-specific [+ ,+ ,0] (magenta) and (F) tail-specific [0,0,+] (green) candidates. Each scatterplot is based on differentially expressed candidates identified through the pairwise *DESeq* comparison of expression in *isolated* worms (*x* axis, mean of \log_2 -transformed expression level for four biologically independent replicates) against the equivalent data for worms in *octets* (*y* axis). Differentially expressed candidates (all FDR < 0.10) for that positional class (in the corresponding colour) are plotted on a background of expression data for all other transcripts (in grey). The numbers at the bottom right of each panel refer to the total number of differentially expressed candidates identified, as the sum of the number that exhibit higher (up arrow) or lower (down arrow) expression in *octets*, respectively, and below that the percentage figure indicates the relative number of differentially expressed candidates compared to the total number of transcripts belonging to that positional class. In each plot (B-F), the red line indicates equal expression in both social conditions, for reference.

Figure 5. Transcriptomic reaction norms for gametogenesis and other aspects of sex allocation in *M. lignano*.

Reaction norms for differentially expressed (A) testis-specific candidates of class [+ ,0,0] (N = 1,053; mean \pm SE slope connecting adjacent treatments, isolated-pairs: 0.63 ± 0.08 ; pairs-octets: 0.35 ± 0.08), (B) ovary-specific candidates of class [0,+ ,0] (N = 125, isolated-pairs: -0.55 ± 0.29 ; pairs-octets: -0.51 ± 0.29), (C) ovary-specific candidates of class [+ ,+ ,0] (N = 80, isolated-pairs: -0.62 ± 0.36 ; pairs-octets: -0.49 ± 0.36) and (D) tail-specific candidates of class [0,0,+] (N = 150, isolated-pairs: 2.24 ± 0.30 ; pairs-octets: 0.59 ± 0.30), illustrating the quantitative nature of sex allocation plasticity. The raw data for each transcript are plotted in colours corresponding to Figure 3, except that reaction norms for transcripts that differed in expression in the opposite-to-prevailing direction (i.e. 20 testis-specific candidates with lower expression in octets, 5 ovary-specific candidates with higher expression in octets, and 10 tail-specific candidates with lower expression in octets) are plotted in black. The mean expression level in each of the three treatments is superimposed for reference (bold black symbols and lines). The numbers refer to the average changes in

expression between adjacent treatments, based on all differentially expressed transcripts belonging to that positional class (see main text for details).

Figure 6. *In situ* hybridization screen for tissue-specific expression of differentially expressed transcripts, examining those with putative testis- or ovary-specific expression.

(A) Ten putatively testis-specific transcripts in class [+O,O] were investigated (plot as in Fig. 3). All but one (I) fit the expected pattern. Three of the four transcripts with higher expression in octets (B-D) are testis-specific (Te) as predicted, while (E) exhibits ISH staining in both testes and (perhaps more weakly) ovaries (Ov). By contrast, the four and two transcripts that had, respectively, lower expression in octets (F-I), or that did not differ in expression between isolated and octets (J-K), exhibit varying ISH patterns, sometimes including but never limited to the testis, instead also including the rostrum (Ro), pharynx (Ph), postpharyngeal glands (Po), gut (Gu), ovaries, developing eggs (De), antrum (An) and/or prostate (Pr). (L) Thirteen putatively ovary-specific transcripts were investigated from classes [O,+,O] and [+,+,O]. Of the ten transcripts expected to be ovary-specific (panels M-O & S-Y), seven indeed exhibit expression limited to the ovary and/or developing eggs (N, S, U, V, W, X and Y; but note in X also single small dots staining in the pharynx/anterior gut region) whereas for the other three, in addition to the ovary, there is also weak staining in the testis or at the testis periphery (M, O, T). Of the three candidates that are not socially sensitive in their expression, one is expressed in the ovary and in developing eggs (P) and two are expressed in both gonads (Q, R).

Figure 7. RNAi knockdown of selected testis- and ovary-specific transcripts reveals functions in gametogenesis pathways.

We knocked down the expression of twelve differentially expressed transcripts that we had confirmed to have testis- or ovary-specific expression using *in situ* hybridization, plus one non-reproductive control transcript known to be expressed exclusively in the adhesive system (*macif1*) (Lengerer *et al.* 2014). We then assessed reproductive phenotypes by examining worms under differential interference microscopy and measuring a standardized suite of traits: (A) body area; (B) testes area; (C) ovaries area; (D) number of developing eggs; and (E) received sperm status (i.e., the presence or absence of received sperm in the antrum). In total, we found nine instances where one of the testis/ovary RNAi knockdown phenotypes differed significantly from the *macif1* control (see main text for details): in panels (A-D), these significant effects are highlighted in red, in (E) by asterisks. Some of the significant treatment effects are illustrated on the right-hand side of the figure. The smaller body size and enlarged testes (Te) in MLRNA110815_14701 knockdown worms (F,G) was accompanied by an unusual sperm morphology, in which many sperm were bent in the shaft region, as indicated by the arrowheads (H). Representative images for four MLRNA110815_1214.1 knockdown worms illustrate the large number of eggs typically present, each highlighted with an arrowhead (I). Compared to controls (J), both MLRNA110815_54 (K) and MLRNA110815_10574 (L) knockdowns can be observed to have large ovaries (Ov).

Supplementary Figure S1. *In situ* hybridization expression patterns for 41 differentially expressed, non-specific candidates.

The transcripts exhibited a wide variety of ISH patterns (summarised in the inset), with specific expression in tissues including the gut (gu), both gonads (go), developing eggs (de), ovaries (ov), pharynx region or pharyngeal glands (ph), rostrum (ro), prostate (pr), epidermis (ep), adhesive system (ad), cement glands (ce), testes (te) and rhabdites (rh). One additional transcript (MLRNA110815_21158.1) exhibited an unspecific ISH staining pattern (not shown). MLRNA110815_21158.2 exhibited different patterns in two different ISH experiments, so the assignment is less certain. The staining in the pharynx region for MLRNA110815_5085 likely corresponds to frontal glands type 3, in MLRNA110815_26830 to weak expression in frontal glands type 1 (with several worms also appearing to exhibit weak expression overall and/or in the gonad) and MLRNA110815_2945.1 to postpharyngeal cells (Lengerer *et al.* 2016). Note that the total sum of expression types shown in the inset is greater than the number of transcripts, because for some transcripts specific expression was found for more than one tissue. Also note that a substantial proportion of these ‘non-specific’ transcripts in fact have reproduction-specific expression (testis, ovary, developing eggs, both gonads or prostate), emphasizing that the earlier positional RNA-Seq classification (Arbore *et al.* 2015) does not provide an exhaustive list of tissue-specific candidates.

Supplementary Files

(see metadata tab in each file for a full description of the data contained therein)

Supplementary File S1

Morphological data collected to test for sex allocation plasticity following the initial group size experiment.

Supplementary File S2

List of primers used for the ISH and RNAi experiments.

Supplementary File S3

Count data, treatment means and differential expression analyses for all 76,437 *M. lignano* transcripts studied, plus corresponding transcript IDs for the MLRNA150904 and Mlig_RNA_3_7_DV1_v3 transcriptome assemblies.

Supplementary File S4

List of all transcripts in the Mlig_RNA_3_7_DV1_v3 transcriptome assembly and their corresponding transcripts in the MLRNA110815 transcriptome assembly.

Supplementary File S5

Full functional annotations from the Mlig_RNA_3_7_DV1_v3 transcriptome assembly for the top 100 plastic transcripts, expanding on data presented in Fig. 2.













